ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1976, p. 696-700

Vol. 9, No. 4
Printed in U.S.A.

Uptake of Metronidazole and Its Effect on Viability in Trichomonads and *Entamoeba invadens* Under Anaerobic and Aerobic Conditions

MIKLÓS MÜLLER* AND DONALD G. LINDMARK
The Rockefeller University, New York, New York 10021

Received for publication 12 December 1975

[^{14}C]metronidazole used at the chemotherapeutic concentration of 10 μg/ml is taken up rapidly by the anaerobic protozoa *Tritrichomonas foetus*, *Trichomonas vaginalis*, and *Entamoeba invadens* kept under anaerobic conditions. It can be calculated that within 30 to 60 min the intracellular concentration of the label is 50 to 100 times higher than in the medium. The presence of air markedly suppresses the uptake in the trichomonads and abolishes it in *E. invadens*. The suppression disappears after anaerobic conditions are established. The rate of uptake in *T. foetus* is dependent on the concentration of the drug in the range studied (1 to 200 μg/ml). Analysis of double reciprocal plots suggests that the drug enters the cells predominantly or exclusively by diffusion. The major factor driving the uptake is most likely the intracellular biotransformation of the compound. If less than 3 μg of drug per mg of protein is taken up by *T. foetus* no decrease in viability is observed. Above this level the cytotoxic activity corresponds roughly to the amount accumulated in the cell, irrespective of whether the conditions are anaerobic or aerobic.

Metronidazole [Flagyl; 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole], as well as several other 5-nitroimidazole derivatives, strongly inhibits anaerobic protozoa and anaerobic bacteria and has very limited biological activity in aerobic organisms (7). Such a high specificity is usually observed only with compounds that selectively inhibit certain biochemical processes in prokaryotic microorganisms without affecting similar processes in eukaryotes. The specificity of 5-nitroimidazole derivatives, however, cuts across the line between prokaryotes and eukaryotes, and thus the most favored explanation of the selectivity is that these drugs interact with biochemical systems or pathways found only in anaerobes (5, 9). The nature and mechanisms of this interaction are not fully understood.

We studied the uptake of the [^{14}C]metronidazole by three anaerobic protozoa and its effect on the viability of one species. In view of the known effect of the presence or absence of O_2 on the metabolism of anaerobic organisms (6, 14, 17, 18), we examined whether O_2 had any effect on the activity of metronidazole. Additional interest in this question is due to the fact that many organisms against which 5-nitroimidazoles are used are present in host tissues that are not anaerobic.

MATERIALS AND METHODS

The following protozoan species were used: the flagellates *Tritrichomonas foetus* (strain KV_1) from cattle and *Trichomonas vaginalis* (ATCC 30001) from humans, parasites of the genito-urinary tract, and the rhizopod *Entamoeba invadens* (ATCC 30020), a parasite of reptile intestine. All three strains were axenic and grown under the following conditions: *T. foetus* for 1 day in Diamond's TYM medium (2) without agar, pH 7.2, with 10% bovine serum at 37 C; *T. vaginalis* for 1 day in the same medium, pH 6.0, with 10% horse serum at 37 C; *E. invadens* for 7 days in Diamond's TPS-1 medium (3), pH 7.0, with 10% bovine serum at 25 C. The media for *T. vaginalis* stock cultures contained 0.1% agar. Material used in the experiments was passaged twice in agar-free medium before inoculating the final cultures.

The cells were collected by centrifugation at room temperature (2,500 rpm for 5 min in the SS-34 rotor of the RC-3 refrigerated centrifuge; DuPont-Sorvall, Norwalk, Conn.), washed twice in a buffered salt solution (4) (pH 6.3 for the trichomonads and pH 6.9 for the entamoeba) under identical conditions, and finally suspended in the same solution.

Samples of appropriate volume (usually 20 ml) were placed in 50-ml Erlenmeyer flasks that were closed with rubber serum stoppers (no. 10-4798; Ace Scientific Supply Co., Inc., Linden, N.J.) and incubated under orbital shaking (200 cycles/min) in a water bath (AquaTherm, New Brunswick Scientific

Co., New Brunswick, N.J.) at 37 C (trichomonads) or 25 C (*E. invadens*). The flasks were flushed throughout the experiments with analyzed gas mixtures containing 5% CO_2 either in N_2 or air (Matheson Co., Rutherford, N.J.). Hypodermic needles inserted into the rubber caps served as gas inlets and vents.

[^{14}C]metronidazole (uniformly labeled in the hydroxyethyl side chain) dissolved in water or in the above-mentioned buffered salt solution (if the injected volume exceeded 1% of that of the sample in the flask) was injected into the flasks after 30-min equilibration with the gas phase. Samples were withdrawn periodically with hypodermic syringes.

To determine the uptake of label by the cells, samples were centrifuged for 1 min at top speed in a tabletop clinical centrifuge (model CL, International Equipment Co., Needham, Mass.). The sedimented cells were washed twice under identical conditions in 250 mM sucrose and finally dissolved in 100 mM NaOH containing 0.4% sodium deoxycholate. Another part of the whole sample was diluted with the same mixture for the determination of label present in the initial suspension (cells and medium together). Radioactivity in aliquots of the dissolved samples was determined by scintillation counting.

Protein content of the dissolved samples was determined by an automated Lowry procedure (10). Bovine serum albumin was used as a standard.

Cells in samples appropriately diluted with the buffered salt solution were counted with a Coulter counter (model B, Coulter Corp., Hialeah, Fla.).

Viability of *T. foetus* was assessed by evaluating growth in serial dilutions according to the method of most probable number. Tubes containing the dilutions made with the complete growth medium were incubated in anaerobic jars (GasPak Anaerobic System, BBL, Cockeysville, Md.) in an H_2 and CO_2 atmosphere (generated by GasPak no. 70304 disposable gas generator envelopes and checked with GasPak no. 70504 disposable anaerobic indicator).

RESULTS

Figure 1 shows the appearance of label in cells if [^{14}C]metronidazole is added at a final concentration of 10 μg/ml. The uptake by all three species is rapid under anaerobic conditions. Under aerobic conditions trichomonad cells still accumulate label, albeit at a much slower rate, whereas uptake by *E. invadens* cells is not detectable. If the conditions are changed from anaerobic to aerobic or the reverse, the rate of uptake is adjusted to the new conditions within a few minutes in the case of trichomonads and somewhat slower in *E. invadens*.

The order of magnitude of the ratio of the drug concentrations in the cells and in the medium was estimated by assuming that proteins represent at least 10 to 20% of the weight of the cells, and thus 1 mg of protein corresponds to an approximately 5- to 10-μl cell volume. An accumulation of 5 μg of metronidazole per mg of protein, attained by all three species within 30 to 60 min under anaerobic conditions, thus corresponds to an intracellular concentration of 500 to 1,000 μg/ml, exceeding the extracellular value by a factor of 50 to 100.

More detailed experiments were performed

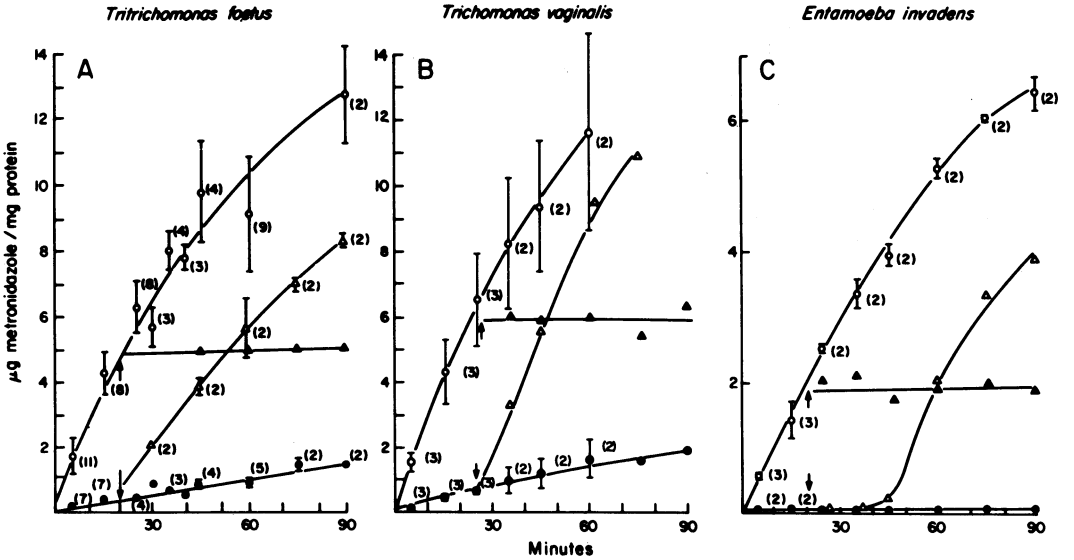

FIG. 1. *Uptake of [^{14}C]metronidazole (10 μg/ml) by T. foetus (A), T. vaginalis (B), and E. invadens (C). The gas phase was 5% CO_2 in air (●) or in N_2 (○). In some experiments the gas phase was changed (solid arrows) from air to N_2 (△) or from N_2 to air (▲). Points shown are mean values ± standard deviation of data obtained in (n) separate experiments.*

on *T. foetus*, the species most amenable to experimentation. The velocity of uptake of label, as defined by the linear portion of the uptake curve between 5 and 25 to 35 min, was determined for different concentrations of metronidazole (1 to 100 μg/ml under N_2 and 2 to 200 μg/ml under air). The uptake is clearly dependent on the drug concentration under both anaerobic and aerobic conditions. Double reciprocal plots of the results (Fig. 2) suggest that the entry of the drug does not follow saturation kinetics within the concentration ranges studied.

Figure 3 shows the change in the number of viable cells of *T. foetus* in the presence of different concentrations of metronidazole under anaerobic and aerobic conditions. In all cases viability decreases roughly logarithmically. As expected from the marked dependence of uptake on the presence or absence of O_2, the drug is much less effective under aerobic conditions. For instance, 10 μg of drug per ml kills more than 99% of the trichomonads in the absence, but none in the presence, of O_2 in 90 min. No decrease in viability was observed in control experiments when *T. foetus* cells were incubated aerobically or anaerobically in the absence of drug.

By appropriate choices of the outside concentration of metronidazole one can easily manipulate the amount of drug taken up by the cells. Uptake of less than 3 μg of protein per mg has no effect, but uptake of larger amounts results in loss of viability (Fig. 4). Although there is much scatter in the results, the degree of killing is dependent on the amount of drug taken up. It is of special interest that about the same loss of viability is observed in aerobic and anaerobic experiments, provided that the same amount of label was taken up.

DISCUSSION

Our results show that the label from [^{14}C]-metronidazole rapidly accumulates in trichomonads and *E. invadens* kept in non-nutritive medium under anaerobic conditions. This is in agreement with earlier experiments in which metronidazole added to growing cultures was taken up by anaerobic protozoa and bacteria but not by the aerobic protozoa *Tetrahymena pyriformis* and *Trypanosoma cruzi* or

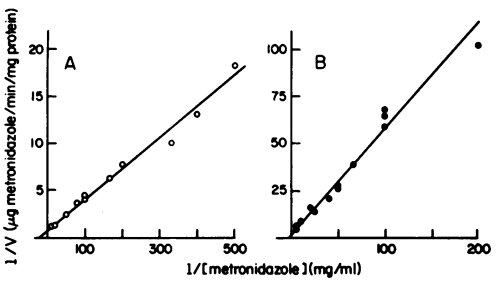

FIG. 2. *Uptake of [^{14}C]metronidazole by T. foetus under N_2 (A) or air (B). Double reciprocal plots.*

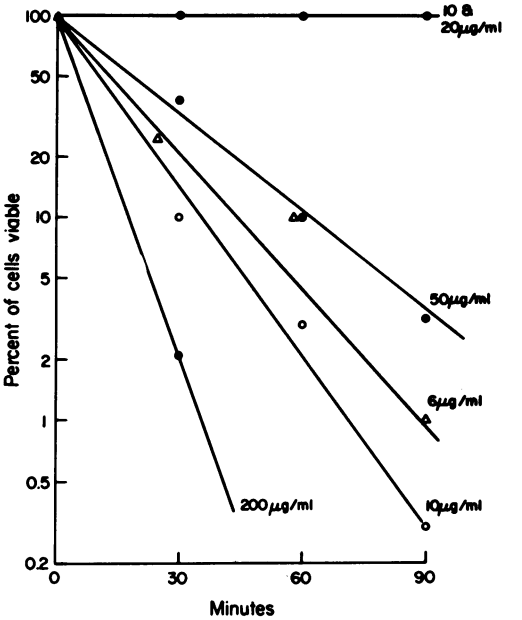

FIG. 3. *Effect of metronidazole in different concentrations on the viability of T. foetus under air (●) or N_2 (○).*

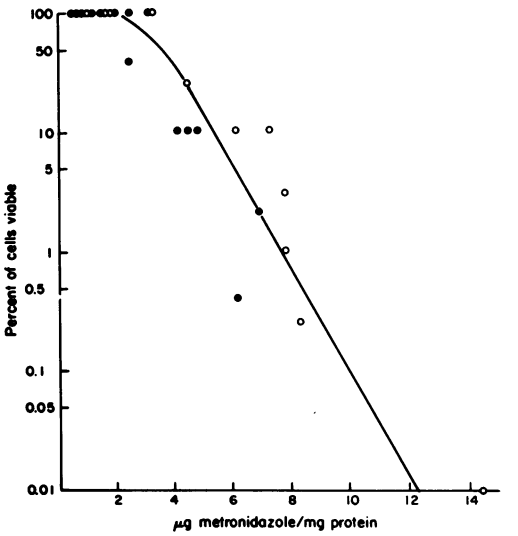

FIG. 4. *Relationship of the amount of ^{14}C label of metronidazole taken up in 25 to 60 min by T. foetus cells and their viability. Experiments under air (●) or N_2 (○).*

by vertebrate cells in tissue culture (9, 20).

The concentration dependency of the uptake process does not indicate a significant participation of mediated processes. If such processes occur, as suggested (9), they possibly do not play an important role in the therapeutic concentration range.

Atmospheric O_2 is a powerful inhibitor of the uptake process. In trichomonads it decreases the rate to low levels, whereas in *E. invadens* it abolishes the uptake. This effect is, however, completely reversible and thus it is not likely to be due to damage to the cells or to sensitive enzyme systems. The presence or absence of O_2 is known to influence markedly the metabolism of anaerobic cells, usually because O_2 is a preferred acceptor of electrons from different pathways. The marked effect of O_2 on metronidazole uptake strongly suggests that the process depends on the availability of reducing power inside the cell.

The role of this reducing power is possibly in modifying the drug by reduction of the 5-nitro group after it enters the cell and thus in keeping the intracellular concentration of the unchanged drug low. This generates a concentration gradient that drives the uptake of the drug and explains the accumulation of large amounts of ^{14}C label in the cell. The occurrence of such a reduction in anaerobic cells has been repeatedly suggested and support for it comes from the findings that extracts of trichomonads and anaerobic bacteria reduce the drug (12, 16) and that, in trichomonads treated with metronidazole, no unchanged drug is found and ^{14}C label introduced with the drug is in compounds that have no nitro group and biological activity (9).

The nature of the intracellular redox system has been the subject of much discussion. It has been suggested that ferredoxin- and flavodoxin-type electron transport proteins are responsible for the reduction of the compounds (5, 16). Reduced ferredoxin is able to reduce metronidazole in a non-enzymatic reaction (D. G. Lindmark, unpublished data). These proteins are known to occur and play a major role in the metabolism of anaerobic prokaryotes and to be of no or only specialized significance in nonphoto synthetic aerobic eukaryotes (15). Although such proteins were not yet directly demonstrated in trichomonads or *Entamoeba* species, the occurrence of a pyruvate synthase and hydrogenase that utilize exogenous ferredoxin (11, 13; D. G. Lindmark, *in* B. Sepúlveda and L. Lauda (ed.), *Proceedings of the International Conference on Amebiasis*, in press), as well as the well-documented H_2 formation in these forms (1, 14, 18), provides strong indirect evidence for their presence and role in metabolism. Accordingly, such low-redox-potential redox systems could represent a major unifying link between those prokaryotes and eukaryotes that are highly sensitive to 5-nitroimidazoles and they are likely to be involved in the reduction of the drug.

The absence or subordinate role of systems able to reduce the nitro group of the drug explains the low uptake and effect of these compounds in aerobic organisms. Mammalian organisms treated with 5-nitroimidazole derivatives excrete most if not all of the drugs unchanged and as derivatives with intact nitro group (8, 19).

The products of the reductive biotransformation of metronidazole have not been characterized (9). It has been suggested that short-lived, highly active products are formed that interact with deoxyribonucleic acid and thus inhibit macromolecular synthesis and exert cytotoxic action (9, 21). In the present work we found that cytotoxicity in *T. foetus* is related only to the amount of label, i.e., drug taken up, and is not inhibited by O_2. This finding suggests that the protective effect of O_2 on metronidazole-treated organisms is primarily due to decreased uptake.

In conclusion, the selective action of 5-nitroimidazoles is best explained by the assumption that intracellular reduction of the nitro group by the anaerobic redox systems of susceptible organisms is essential for two reasons. It decreases the intracellular concentration of the unchanged drug and thus maintains a gradient driving the uptake, and it generates a possibly short-lived compound that is toxic to the cell. The rate of this process depends on the competition for the reducing power. As shown, O_2 is a very powerful competitor in this process. It is a likely possibility that the chemotherapeutic efficiency of the 5-nitroimidazoles will be different against infections localized in tissues with high or low pO_2 values. In addition the present experiments suggest that labeled metronidazole might be a useful tool in the assessment of the functioning of the low-redox-potential systems in different anaerobic microorganisms.

ACKNOWLEDGMENTS

Cold and ^{14}C-labeled metronidazole was obtained from G. D. Searle & Co., San Juan, Puerto Rico, and Searle Laboratories, Chicago, Ill., through the courtesy of F. McIlreath, S. Kraychy, and E. Roman. The KV, strain of *T. foetus* was kindly sent by B. M. Honigberg, Amherst, Mass.; strains of the two other species were recieved from the American Type Culture Collection, Bethesda, Md.

This work was supported by Public Health Service grant

AI-11942 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Bauchop, T.** 1971. Mechanism of hydrogen formation in *Trichomonas foetus*. J. Gen. Microbiol. 68:27–33.
2. **Diamond, L. S.** 1957. The establishment of various trichomonads of animals and man in axenic cultures. J. Parasitol. 43:488–490.
3. **Diamond, L. S.** 1968. Techniques of axenic cultivation of *Entamoeba histolytica*-like amoebae. J. Parasitol. 54:1047–1051.
4. **Doran, D. J.** 1959. Studies on trichomonads. III. Inhibitors, acid production, and substrate utilization by 4 strains of *Tritrichomonas foetus*. J. Protozool. 6:177–182.
5. **Edwards, D. I., M. Dye, and H. Carne.** 1973. The selective toxicity of antimicrobial nitroheterocyclic drugs. J. Gen. Microbiol. 76:135–145.
6. **Gray, C. T., and H. Gest.** 1965. Biological formation of molecular hydrogen. Science 148:186–192.
7. **Grunberg, E., and E. Titsworth.** 1974. Chemotherapeutic properties of heterocyclic compounds: monocyclic compounds with five membered rings. Annu. Rev. Microbiol. 27:317–347.
8. **Ings, R. M. J., G. L. Law, and E. W. Parnell.** 1965. The metabolism of metronidazole (1-2′-hydroxyethyl-2-methyl-5-nitroimidazole). Biochem. Pharmacol. 15: 515–519.
9. **Ings, R. M. J., J. A. McFadzean, and W. E. Ormerod.** 1974. The mode of action of metronidazole in *Trichomonas vaginalis* and other microorganisms. Biochem. Pharmacol. 23:1421–1429.
10. **Leighton, F., B. Poole, H. Beaufay, P. Baudhuin, J. W. Coffey, S. Fowler, and C. de Duve.** 1968. The large scale separation of peroxisomes, mitochondria and lysosomes from the livers of rats injected with Triton WR-1339. Improved isolation procedures, automated analysis, biochemical and morphological properties of fractions. J. Cell Biol. 37:482–513.
11. **Lindmark, D. G., and M. Müller.** 1973. Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Tritrichomonas foetus* and its role in pyruvate metabolism. J. Biol. Chem. 248:7724–7728.
12. **Lindmark, D. G., and M. Müller.** 1974. Reduction of metronidazole by homogenates of trichomonad flagellates. J. Protozool. 21:436.
13. **Lindmark, D. G., M. Müller, and H. Shio.** 1975. Hydrogenosomes in *Trichomonas vaginalis*. J. Parasitol. 61:552–554.
14. **Montalvo, F. E., R. E. Reeves, and L. G. Warren.** 1971. Aerobic and anaerobic metabolism in *Entamoeba histolytica*. Exp. Parasitol. 30:249–256.
15. **Mortenson, L. E., and G. Nakos.** 1973. Bacterial ferredoxins and/or iron-sulfur proteins as electron carriers, p. 37–60. *In* W. Lovenberg (ed.), Iron-sulfur proteins, vol. I. Academic Press Inc., New York.
16. **O'Brien, R. W., and J. G. Morris.** 1972. Effect of metronidazole on hydrogen production by *Clostridium acetobutylicum*. Arch. Mikrobiol. 84:225–233.
17. **Read, C. P., and A. H. Rothman.** 1955. Preliminary notes on the metabolism of *Trichomonas vaginalis*. Am. J. Hyg. 61:249–260.
18. **Ryley, J. F.** 1955. Studies on the metabolism of protozoa. 5. Metabolism of the parasitic flagellate *Trichomonas foetus*. Biochem. J. 59:361–369.
19. **Stambaugh, J. E., L. G. Feo, and R. W. Manthei.** 1968. The isolation and identification of the urinary oxidative metabolites of metronidazole in man. J. Pharmacol. Exp. Ther. 161:373–381.
20. **Tanowitz, H. B., M. Wittner, R. M. Rosenbaum, and Y. Kress.** 1975. In vitro studies on the differential toxicity of metronidazole in protozoa and mammalian cells. Ann. Trop. Med. Parasitol. 69:19–27.
21. **Wilson, R. L., and A. J. F. Searle.** 1975. Metronidazole (Flagyl): iron catalyzed reaction with sulphydryl groups and tumour radiosensitization. Nature (London) 255:498–500.